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The Genetics of Fertility in Soybean

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Abstract
Male and female reproductive structures play an important role in seed development in plants. Abnormalities in male or female reproductive structures can lead to sterility. In soybean, Glycine max (L.) Merr., about 75 sterility mutants have been identified and most of them have been mapped to chromosomes. Mapping results have shown that some chromosomal regions are hotspots for fertility genes. Fine mapping of some of the male-sterile, female-fertile mutants and male-sterile, femalesterile mutants resulted in identification of candidate genes for fertility. Sequence comparisons further helped in locating a few putative candidates. A CACTA- like transposable element that is responsible for reversion from sterility-to-fertility has been identified, and complete association between the presence of a transposon and sterility also has been shown. Several studies are underway that are using transformation sequences to clone fertility genes. Cloning and characterization of genes involved in male sterility and female sterility will help us recognize molecular mechanisms controlling sterility and help us understand the reproductive biology of soybean. This advancement of knowledge will assist in the development of a stable sterility system in soybean that can be utilized for hybrid seed production.

Keywords
Molecular mapping, revertants, soybean, sterility, transposable element

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The Genetics of Fertility in Soybean

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Abstract

Male and female reproductive structures play an important role in seed development in plants. Abnormalities in male or female reproductive structures can lead to sterility. In soybean, *Glycine max* (L.) Merr., about 75 sterility mutants have been identified and most of them have been mapped to chromosomes. Mapping results have shown that some chromosomal regions are hotspots for fertility genes. Fine mapping of some of the male-sterile, female-fertile mutants and male-sterile, female-sterile mutants resulted in identification of candidate genes for fertility. Sequence comparisons further helped in locating a few putative candidates. A CACTA- like transposable element that is responsible for reversion from sterility-to-fertility has been identified, and complete association between the presence of a transposon and sterility also has been shown. Several studies are underway that are using transformation sequences to clone fertility genes. Cloning and characterization of genes involved in male sterility and female sterility will help us recognize molecular mechanisms controlling sterility and help us understand the reproductive biology of soybean. This advancement of knowledge will assist in the development of a stable sterility system in soybean that can be utilized for hybrid seed production.

Key words: Molecular mapping, revertants, soybean, sterility, transposable element

Nuclear sterility mutations in self-compatible plants, such as soybean *Glycine max* (L.) Merr., can be classified as synaptic, structural, male-partial sterile or female-partial sterile, male sterile and female sterile, and male sterile and female fertile (Johns et al. 1981; Kaul 1988). In soybean, several meiotic mutants have been described genetically and cytologically (Table 1). Several of these mutants have been mapped to Classical Linkage Groups (GLGs) and Molecular Linkage Groups (MLGs) (Table 1).

Table 1. Sterility mutations in soybean with gene symbols and/or locations

			Locations[1]		
Gene	**Phenotype**	**Frequency**	**CLG**	**MLG**	**Reference**
fs1fs2	Structural sterile	1			Johns and Palmer (1982)
Fsp1	Female-partial sterile	1	02	D1b	Kato and Palmer (2003a)
Fsp2	Female-partial sterile	1	06	C2	Kato and Palmer (2004)
Fsp3	Female-partial sterile	1	08	A2	Kato and Palmer (2004)
Fsp4	Female-partial sterile	1	13	F	Kato and Palmer (2004)
Fsp5	Female-partial sterile	1	18	G	Kato and Palmer (2004)
ft	Structural sterile	1			Singh and Jha (1978)
ms1	Male sterile, female fertile	6	13	F	Palmer et al. (2004)
ms2	Male sterile, female fertile	2	10	O	Cervantes-Martinez et al. (2007)
ms3	Male sterile, female fertile	3	02	D1b	Cervantes-Martinez et al. (2009)
ms4	Male sterile, female fertile	2			Palmer et al. (2004)
ms5	Male sterile, female fertile	1			Buss (1983)
ms6	Male sterile, female fertile	2	13	F	Palmer et al. (2004)
ms7	Male sterile, female fertile	1			Palmer (2000)
ms8	Male-partial sterile	1	07	M	Frasch et al. (2011)
ms9	Male sterile, female fertile	1	03	N	Cervantes-Martinez et al. (2007)
*ms*MOS	Male sterile, female fertile	1	02	D1b	Jin et al. (1998)
msp	Male-partial sterile	1	02	D1b	Frasch et al. (2011)
st2	Asynaptic male and female sterile	1			Hadley and Starnes (1964)
st3	Asynaptic male and female sterile	1			Hadley and Starnes (1964)
st4	Desynaptic male and female sterile	1			Palmer (1974)
st5	Desynaptic male and female sterile	1	13	F	Palmer and Kaul (1983)
st6st7	Male sterile, female sterile	1			Ilarslan et al. (1997)
st8	Desynaptic male and female sterile	39	16	J	Kato and Palmer (2003b); Palmer et al. (2008); Slattery et al. (2011); Raval et al. (2012)
T-DNA	Male sterile, female sterile	1	01	D1a	Baumbach et al. (2012)
no symbol	Male sterile, female sterile	3	14	B2	Slattery et al. (2011)
no symbol	Male sterile, female sterile	1	02	D1b	Slattery et al. (2011)
no symbol	Male sterile, female sterile	1	18	G	Slattery et al. (2011)
no symbol	Male sterile, female sterile	1	18	G	Palmer et al. (2008)

[1]CLG= classical linkage group designation

[1]MLG= molecular linkage group designation

Sterility mutations as a source of aneuploids

Aneuploids are individuals with other than an exact multiple of the haploid chromosome number. Aneuploids have been observed among progeny of triploids and plants homozygous for certain meiotic abnormalities (Khush 1973). In soybean, aneuploids have been identified, at a low frequency, among progeny of asynaptic and desynaptic mutants. Seeds (pods) have been observed on desynaptic *st4 st4* plants. Table 2 summarizes data on chromosome number, pollen fertility, and number of seeds of progeny from desynaptic segregates in mutant *st4 st4*. Progeny plants that had high levels of male and female fertility, most likely had the male gamete (*st4*) come from fertile sibling (*St4 St4*) plants via insect-mediated cross-pollinations. This was confirmed when progeny of fertile 40- and 41-chromosome plants segregated 3 fertile: 1 sterile plants in the next generation.

Table 2. Chromosome numbers, pollen fertility, and number of seeds of the progeny from desynaptic soybean segregates in mutant *st4 st4* plants (1972 data)

Chromosome no.	No. plants	Fertile pollen (%)		No. seeds	
	Fertile-Sterile-Died	Range	Mean	Range	Mean
40	3-1-0	86-91	88	403-929	676
41	12-3-1	50-94	73	61-690	388
42	17-5-4	36-95	75	20-746	260
43	4-1-0	67-74	69	8-46	33
44	5-5-2	18-65	38	13-194	60
45	1-0-1	53	53	16	16
46	0-0-1				
ca 80	0-7-1				

Aneuploids used in mapping studies

Soybean primary trisomics have been used to locate genes on chromosomes. (Honeycutt et al. 1990; Hedges and Palmer 1991; Xu et al. 2000; Zou et al. 2003a, 2003b). Segregation data were standardized by using the observed disomic ratios (self-pollinating progeny of 40-chromosome plants), rather than the theoretical ratios for comparison with the observed trisomic ratios (self-pollinated progeny of 41-chromosome plants). Subsequently, all 20 primary trisomics have been identified and have been assigned to their respective chromosomes (Table 3) (Xu et al. 2000). Eleven molecular linkage groups have been assigned to 11 soybean chromosomes using primary trisomics (Zou et al. 2003).

Table 3. Number, physical length, and percentage heterochromatin of the 20 soybean pachytene chromosomes, with primary trisomic designation, location of qualitative traits and Molecular Linkage Group designations.

Pachynema chromosomes[1]				Association of molecular linkage groups to chromosomes		
Chromosome		Heterochromatin	Primary trisomics	Qualitative traits	MLGs	MLGs[2]
No.	μm	(%)				cM
1	39.79	37.70	Triplo 1	-	D1a	98.41
2	38.12	14.22	Triplo 2	-	D1b	140.63
3	37.71	15.49	Triplo 3	*y10*	N	99.51
4	36.67	49.99	Triplo 4	*dia1*	C1	112.32
5	33.34	22.50	Triplo 5	*v2, eu1*	A1	86.75
6	31.87	56.20	Triplo 6	-	C2	136.51
7	28.75	59.44	Triplo 7	-	M	135.15
8	27.71	26.34	Triplo 8	-	A2	146.67
9	27.08	26.92	Triplo 9	*fr1*	K	99.60
10	26.67	43.76	Triplo 10	-	O	132.89
11	25.42	27.85	Triplo 11	-	B1	124.24
12	24.80	43.67	Triplo 12	-	H	120.50
13	23.55	16.82	Triplo 13	*w1, lx1*	F	120.03
14	20.21	34.04	Triplo 14	-	B2	108.18
15	17.50	57.14	Triplo 15	-	E	99.88
16	15.83	30.32	Triplo 16	-	J	92.27
17	15.83	46.11	Triplo 17	-	D2	119.19
18	14.37	53.65	Triplo 18	-	G	105.00
19	13.54	41.58	Triplo 19	-	L	101.14
20	10.63	47.04	Triplo 20	*p2*	I	112.77

[1]Singh, R. J., and Hymowitz, T. 1988.

[2] Assignment of MLGs to chromosomes based on genetic length (http://soybase.org/LG2Xsome.php). Physical chromosome length measured in μm and MLGs are in cM.

Sterility mutations are an asset in the study of reproductive biology

Detailed light and electron microscopic studies have given us information on microsporogenesis, microgametogenesis, megasporogenesis, and megagametogenesis (Horner and Palmer, 1995). Figure 1 depicts general anther morphology and anatomy showing stamen (anther and filament) and segments of four microsporangia and a single microsporangium with its somatic and male cells and tissues. In Figure 2, microsporogenesis encompasses stages 1-7, and microgametogenesis stages 8-10. Note that mutations at any of these stages can result in partial or complete male sterility.

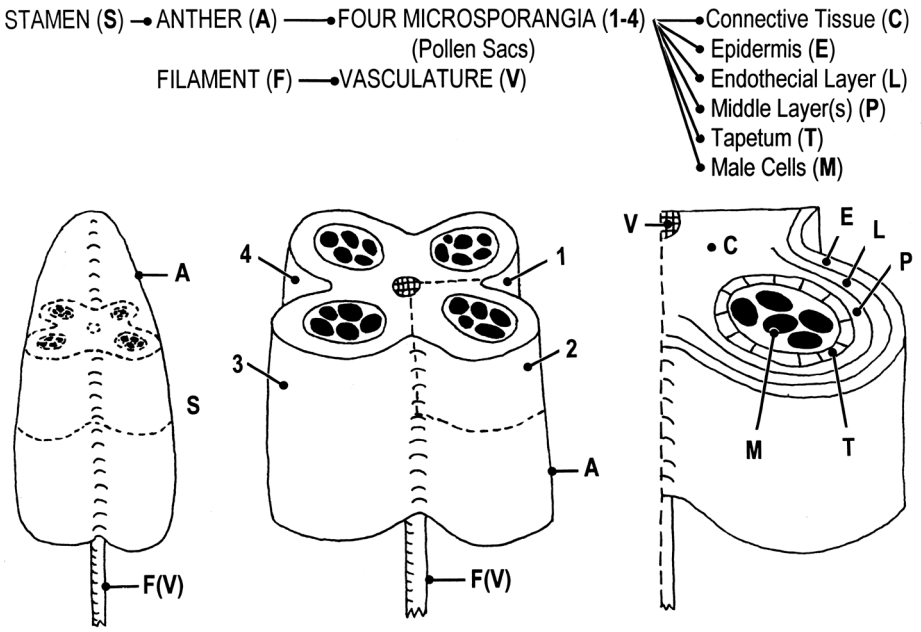

Figure 1. General anther morphology and anatomy showing stamen (anther and filament) segments of four microsporangia and a single microsporangium with its included somatic and male cells and tissues.

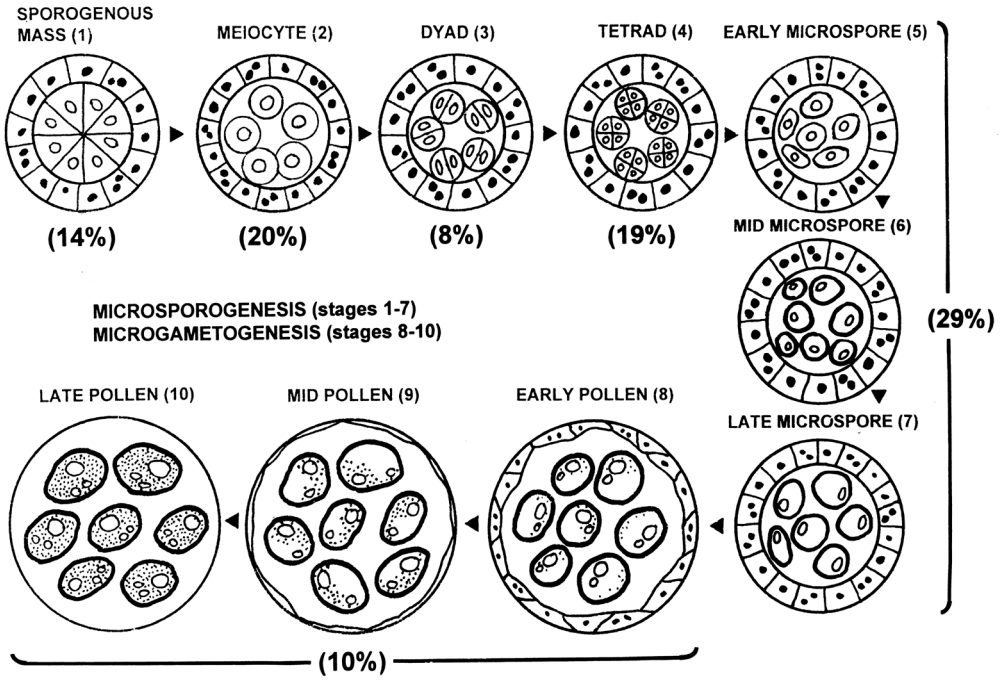

Figure 2. Ten stages of anther development emphasizing male cells and surrounding tapetum, beginning with sporogenous stage (1) and ending with late pollen stage (10). Timing of *ms* gene action (in percent) during anther development (adapted from Horner and Palmer, 1995). Percents in parentheses indicate what percentages of all *ms* steriles have been identified at each stage or series of three stages (adapted from Kaul, 1988).

Microsporogenesis and microgametogenesis

Reproductive development of many of the soybean reproductive biology mutant genotypes have been completed. Figure 3 shows pollen development in fertile and *st4 st4* (desynaptic mutant) plants. Tetrad formation (Figure 3A) and mature pollen (Figure 3C) were normal in the fertile plants. Micronuclei often were present at the tetrad stage (Figure 3B) in desynaptic plants. Restitution nuclei occasionally were seen in the desynaptic mutant. Pollen grains varied greatly in size (Figure 3D), and this observation is consistent with the desynaptic appearance of the chromosomes (Palmer 1974). In Figure 3D, one pollen grain (b) has four germ pores, characteristic of diploid pollen from tetraploid soybean plants (Sen and Vidyabhusan 1960). Pollen from fertile plants and from desynaptic plants were classified for stainability using I_2kI.

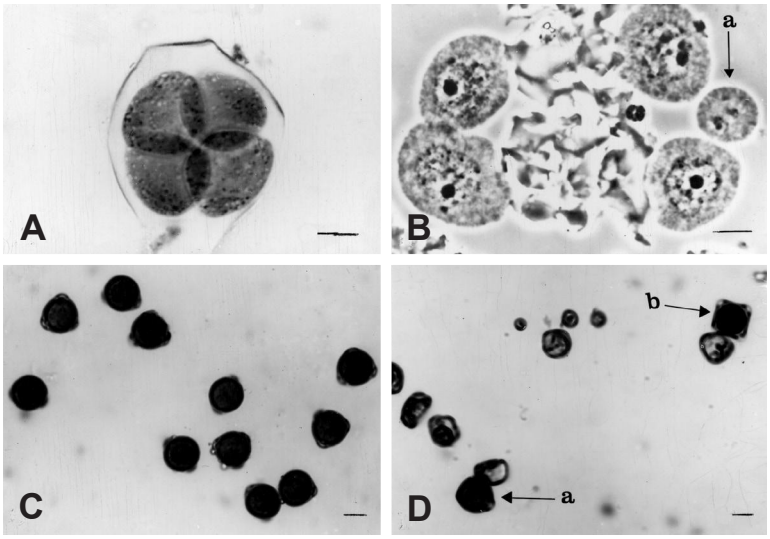

Figure 3. Pollen development in fertile and desynaptic soybean plants. A- tetrad of spores from fertile plant. B- phase contrast picture of four spores and a micronucleus (a) from desynaptic plant. C- pollen from fertile plant, stained with I_2kI; note normal-appearing pollen (a), with four germ pores (b), and small, collapsed, poorly stained pollen. Line scales represent 10 μm.

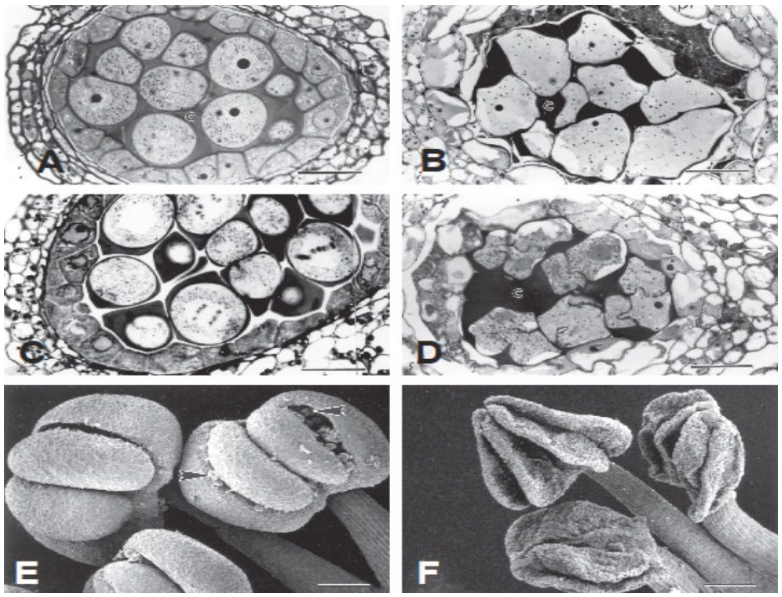

Figure 4. Light microscopy micrographs showing early stages of microsporogenesis and mature anthers in fertile and male-sterile, female-fertile *ms6 ms6* plants. A- Fertile early meiocyte stage. B- fertile late meiocyte stage. C- sterile early meiocyte stage; tapetum is already degenerated and parietal layer is enlarged radially. D- sterile early tetrad stage with partially septated tetrads, a degenerated tapetum, and enlarged parietal layer. E- fertile anthers at anthesis. Anthers are split longitudinally displaying pollen (arrowheads). F- sterile anthers are shriveled, split open sporadically, but do not display any pollen. Line scales represent 200 μm in A,B,C, and D. Line scales represent 100 μm in E and F.

Figure 4 shows light micrographs of early stages of microsporogenesis and mature anthers in fertile and male-sterile, female-fertile *ms6 ms6* plants. During meiosis, fertile plants (Fig. 4A and 4C) formed tetrads of microspores in callose, whereas male-sterile plants (Fig. 4B and 4D) displayed degeneration of the tapetum and a grossly enlarged parietal layer. Callose formed around meiotic cells of male-sterile plants, but these cells did not completely form tetrads (Fig. 4D) because they too degenerated and collapsed as a result of further enlargement of the parietal layer and the disintegrated tapetum.

Anthers from fertile plants near anthesis, observed with the scanning electron microscope (Fig. 4E), were robust at the time they split open to release their pollen. Anthers from open flowers of male-sterile plants (Fig. 4F) were shriveled with no evident pollen.

Use of sterility mutations for hybrid development

Cytoplasmic male sterility, with nuclear restoration and maintainer genes, has been identified in soybean (Palmer et al. 2011). Commercial production of hybrid seed has not been achieved. The high cost of the F_1 seed is the obstacle. Seed-set on male-sterile plants is a strong indicator of insect pollinator preference. Ortiz-Perez et al. (2008) reported that after three backcross generations, the mean out-crossed seed number on male-sterile plants was as high as 232 (Table 4); (Palmer et al. 2009), with the same parental combinations, observed seed-set as high as 242 after derived five-way crosses (Table 4). Thus, the use of phenotypic recurrent selection via insect-mediated cross-pollination was successful.

Table 4. Seed-set from fertile-female soybean parents-derived BC3 crosses compared in percent relative to their fertile-female parent. Texas 2005.

Fertile-female parent	Mean no. seed/ fertile-female parent	Fertile-female parents-derived BC3 crosses	Mean no. seed/ male-sterile line	% seed-set relative to fertile-female parent
A00-41 *Ms2*	219	A00-41 *ms2* × A00-73 (*Ms9*)	136	62
A00-63 *Ms2* (Beeson)	231	A00-63 *ms2* (Beeson) × Wells	99	43
A00-68 *Ms3*	287	A00-68 *ms3* × A00-41 (*Ms2)*	232	80

However, the mechanism(s) for the 'discovery' of the plants by insect-pollinators remains elusive. Our current studies focus on soybean-insect pollinator interaction. The Proboscis Extension Response (PERs) system is used to determine if bees detect differences among floral volatiles emitted by different genotypes. In addition, volatiles from these same genotypes were identified by using gas chromatography - mass spectrometry – olfactometry (Pappas et al. 2012). Soybean floral volatiles are linked to seed-set, with bees remembering the difference between high and low seed-set plants. This difference can most likely be attributed to differences in the relative concentrations of floral volatiles emitted by different soybean genotypes.

Table 5. Seed-set from fertile-female soybean parents-derived five way crosses compared in percent relative to their fertile-female parent. Texas 2005.

Fertile-female parent	Mean no. seed/ fertile -female parent	Derived soybean plants	Mean no. seed/ male-sterile line	% seed-set relative to fertile-female parent
A00-41 *Ms2*	219	A00-41 *ms2* × A00-73 (*Ms9*)	217	99
A00-63 *Ms2* (Beeson)	231	A00-63 *ms2* × Wells	137	59
A00-68 *Ms3*	287	A00-68 *ms3* × A00-41 (*Ms2)*	234	81

Characterizing male-sterile female-fertile mutants

In soybean, identification of an environmentally stable male-sterility system could make hybrid seed production commercially valuable. An environmentally sensitive male-sterile, female-fertile mutant (*ms8*) has been identified. Inheritance studies showed that sterility in the mutant is inherited as a single gene. We used Bulked Segregant Analysis (BSA) to genetically map the gene to MLG M (chromosome Gm07) (Figure 5). The *ms8* is flanked by a telomere and Sat_389 (Frasch et al. 2011). The region between the telomere and Sat_389 is physically 160 Kb.

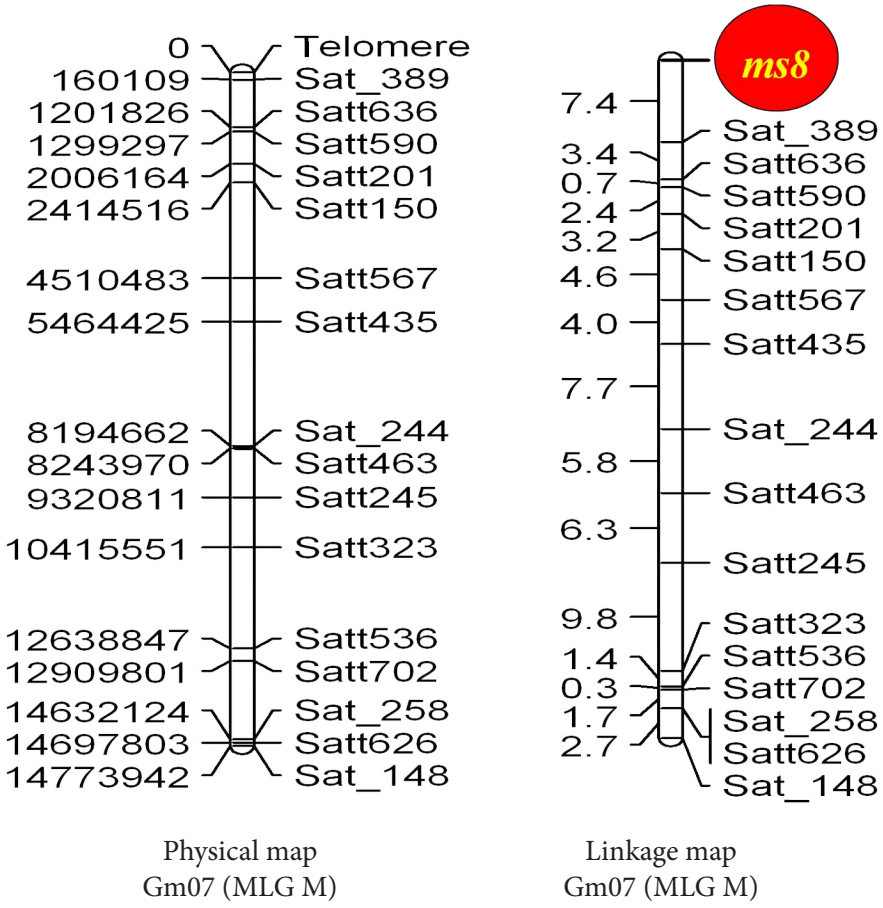

Figure 5. Genetic linkage maps and sequence based on physical maps of soybean chromosome 07 (MLG M) showing locations of SSR markers close to the *ms8* gene. Male-sterile *ms8* locus, Minsoy (*Ms8 Ms8*) x *Ms8 ms8* (adapted from Frasch et al. 2011).

The soybean genome has been sequenced and can be accessed at the Phytozome website (Schmutz et al. 2010; http://www.phytozome.net/). Putative genes in the region of the *ms8* (Frasch et al. 2011), and *ms9* (Wiebbecke, 2011) were located. Thirteen genes were located in the region of *ms8* and nine were located in the region of *ms9*. Protein BLASTP (basic local alignment search tool protein) was used to compare protein sequences against non-redundant protein databases to find homologous sequences and to determine putative functions. BLASTP analysis revealed that homologs of 3 of the 13 genes were known to play a role in cell division, suggesting putative candidates for *ms8* (Frasch et al. 2011). Sequence homology to known male-sterility genes and temperature-dependent expression identified two genes as the most likely candidates for *ms9* (Wiebbecke 2011).

Characterizing male-sterile female-sterile mutants

Several male-sterile female-sterile (MSFS) mutants had been identified in soybean (Table 1). We identified a male-sterile female-sterile (MSFS) mutant in the progeny of *w4*-mutable (*w4*-m) line and named it as *st_A06-321*. The sterility gene shows monogenic inheritance. As both micro- and mega-gametogenesis are affected, action of the *st_A06-321* gene product, most likely, precedes the differentiation of male and female reproductive organs. We mapped the *st_A06-321* gene to chromosome Gm16 (MLG J) (Figure 6; Raval et al. 2012). The location of the *st_A06-321* in relation to the linked markers indicated that *st_A06-321* and *st8* are most likely, allelic (Kato and Palmer 2003; Palmer et al. 2008a; Ravel et al. 2012; Slattery et al. 2011).

Table 6. Genes located in the *st8* region. Putative proteins encoded by five genes that are flanked by BARCSOYSSR_16_0428 and BARCSOYSSR_16_0430 markers on soybean chromosome 16 (MLG J) are shown.

Gene	Putative protein	Predicted function
Glyma16g07850.1	DNA/RNA helicase MER3/ SLH1; DEAD-box superfamily	Meiotic recombination and cytokinesis
Glyma16g07870.1	Gluteradoxin	Catalyzing disulfide bond reduction
Glyma16g07890.1	Unknown	Transcription-related
Glyma16g07910.1	Polynucleotidyl transferase, ribonuclease H domain	Cleaves the RNA strand of an RNA/DNA complex
Glyma16g07920.1	Histone deacetylase	Histone deacetylase

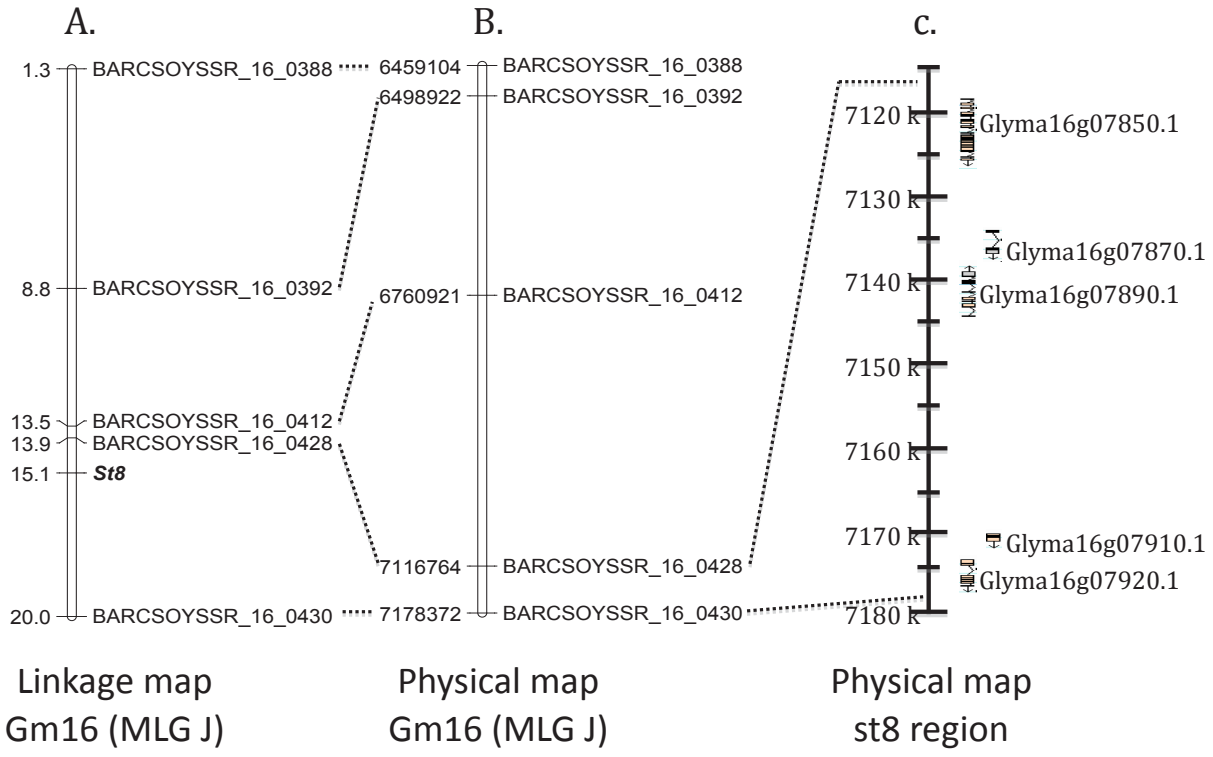

Figure 6. Genetic linkage map and sequence-based physical map of the soybean chromosome Gm16 (MLG J) showing locations of SSR markers close to the *st8* gene. **A.** Genetic map position of the *st8* gene. **B.** Sequence based physical map of Gm16 (Schmutz et al. 2010). C. Chromosomal region flanked by BARCSOYSSR_16_0428 and BARCSOYSSR_16_0430 showing the locations of the predicted genes. Physical distances are shown in base pairs (bp) and genetic distances are shown in centi-Morgans (cM).

Fine mapping of the *st8* gene showed that it is flanked by BARCSOYSSR_16_0428 and BARCSOYSSR_16_0430. Comparison with the soybean genome revealed that these markers are only ~62 Kb apart and there are only five predicted genes present in this region (Figure 6; Table 6; http://www.phytozome.net/). We annotated the five genes by searching their possible similarities to genes with known function using BLASTP program of the National Center of Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The *Glyma16g07850.1* gene codes for a DNA/RNA helicase/DEAD-box protein and was identified as a candidate gene for soybean fertility (Raval et al. 2012). Orthologous proteins in rice, Arabidopsis and yeast are known to play a critical role during the cell cycle (Borner et al. 2004; Mercier et al. 2005; Wang et al. 2009). Transcriptomics analysis revealed that *Glyma16g07850.1* expresses selectively in the apical meristem and root tip, confirming involvement of this protein in the cell cycle (Raval et al. 2012).

Raval et al. (2012) also showed a perfect association between absence/presence of a CACTA-like transposable element (*Tgm9*) and fertility/sterility phenotype in the progenies. This indicated that most likely presence of *Tgm9* is causing sterility phenotype in this mutant. *Tgm9* has been cloned and is shown to be very active transposon with a high frequency of excision. We are using *Tgm9* sequence information to clone the *st8* gene (D. Sandhu, M.K. Bhattacharyya, and R. Palmer, unpublished results). In the near future, other fertility mutants that arose from the progenies of *w4*-m line, can be utilized to clone additional fertility genes. Characterizing and cloning these genes will provide vital molecular insights on the reproductive biology of soybean and other plants, which may become instrumental in exploiting these genes for commercial applications.

Conclusions

Disruptions in microsporogenesis and microgametogenesis can occur at any developmental stage and result in partial or complete male and/or female sterility. These mutants can be used to identify aneuploids, which can aid in mapping mutate genes to chromosomes. Sterility mutants are excellent genetic lines to compare reproductive processes with normal development. Genetic and molecular approaches helped in identification of chromosomal regions containing fertility genes. Many of the soybean sterility mutants occurred spontaneously or were the result of a CACTA transposon. With the cloning of this transposon, characterization and cloning of fertility genes can be expedited. As the possibility of commercial soybean hybrids depends upon a reliable sterility system, understanding the biology of genes involved in soybean reproduction may facilitate commercialization of soybean hybrids.

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